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[RAPID COMMUNICATION]

Identification of Protein C in Sera of the Frogs, *Rana nigromaculata* and *Rana brevipoda*

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ABSTRACT—When sera from the frogs, *Rana* (*R.*) *nigromaculata* and *Rana* (*R.*) *brevipoda*, were run on starch-gel electrophoresis (SGE), several bands were seen in an electrophoretic pattern of proteins. This pattern on SGE appeared the same at stages XIV, XV and XXI, and in the adult frog, *R. niguromaculata*. However, the pattern at stage X was different. A protein, designated "protein C", did not appear clearly at this stage, but afterwards. This protein was the second richest among serum proteins of mature frogs. Protein C (Mr=180 kD, when estimated by SDS-PAGE) was obtained after SGE and then subjected to an NH_2-terminal sequence analysis. Sequences of protein C from *R. nigromaculata* and *R. brevipoda* were NH_2-TDPMYVIFIPQTLXE for the first 15 amino acids and NH_2-TDPHYVIFKG for the first 10 amino acids, respectively. Homology search of GenBank sequences indicated no significant similarity with any known proteins. The results suggest that protein C is a new protein, and that it may play an important role(s) in the serum after stage X in these species.

INTRODUCTION

In order to understand the process of inheritance of a number of characters in amphibians, it is necessary to determine a locus of each gene in the chromosomes. Only a few studies of amphibians have focused on this topic so far [4–6]. This is probably due to difficulties in matching characters with the chromosomes carrying genes. However, by comparison of an electrophoretic pattern of proteins with constitution of bivalent chromosomes in oocytes of female backcrosses among Japanese pond frogs, it is possible to determine which chromosome is carrying a gene for each protein. Nishioka *et al.* [5, 6] determined the loci of five albino genes and 23 genes controlling 3 blood proteins and 13 enzymes on the chromosomes of mature offspring produced from female hybrids between *R. nigromaculata* and *R. brevipoda*, and male parents. They also showed that the gene of one of three blood proteins, designated "protein C", is located on chromosome No. 9. When proteins in sera from two species, *R. nigromaculata* and *R. brevipoda*, were run on starch gel-electrophoresis (SGE) and stained with amido black, several distinct bands were seen. The richest protein was albumin [5], but other proteins including protein C have not been identified yet. To clarify the relationship between a gene and a character, identification of protein C is essential. This study was undertaken to determine developmental change of protein C in the serum and to identify this protein in the frogs, *R. nigromaculata* and *R. brevipoda*.

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MATERIALS AND METHODS

Experimental animals

Mature male frogs, *R. nigromaculata* and *R. brevipoda* collected in the Hiroshima district and the Okayama district, respectively, and female hybrids between these two species were used. For electrophoretic analysis of serum proteins, only offsprings from a pair of *R. niguromaculata* were used. The ovulation of a mature female was induced by bull frog pituitaries. Fertilization was then carried out artificially. Tadpoles in metamorphic stages and mature frogs were fed on boiled spinach or two-spotted crickets [7]. The developmental stage of tadpoles was determined according to Taylor and Kollros [8].

Starch-gel electrophoresis (SGE)

SGE was carried out as previously described [5]. In order to obtain blood samples, 0.1 ml of Ringer's solution containing 200 units of heparin was injected into frog's body cavities. After anesthetization with ether for mature frogs, or with MS-222 [0.01% (w/v); Sankyo] in H_2O for tadpoles, laparotomy was conducted. Blood was taken from the heart with a syringe and subjected to centrifugation at 600×g for 3 min at room temperature to remove blood cells. Sera thus obtained were stored at −20°C until use. For SGE each serum was absorbed in a small piece of filter paper (Whatman, No. 3; 3×7 mm) and placed into a well in a 12% starch-gel (16×18 cm, 6 mm thick) produced in a buffer (A) containing 20 mM boric acid, 0.68 mM EDTA and 21 mM Tris-HCl (pH 8.0). Proteins were run

on SGE at 18.8 V/cm for 4 hr at 2°C in the 10× A (pH 8.0). After electrophoresis, each gel was cut into two slices. One slice was stained with amido black 10B [1% (w/v), Sigma] to identify the spot to which protein C migrated. The other was left unstained to extract protein C. Spots retaining protein C were cut off and boiled for 5 min in 1 ml of a 2% SDS sample buffer [2]. Boiled samples were centrifuged at 5000×g for 10 min at room temperature (Kubota 1900). The resultant supernatant was used to determine the heterogeneity of proteins on SDS-PAGE using a 12% polyacrylamide gel and an NH_2-terminal sequence analysis.

SDS-PAGE

Proteins were heat-denatured in SDS sample buffer and electrophoresed on a discontinuous 12% acrylamide gel [2].

NH_2-terminal sequence analysis

Protein C extracted from starch gels with a 2% SDS sample buffer [2] was prepared for NH_2-terminal sequence analysis using a sample preparation cartridge (ProSpin™; Applied Biosystems) by a protocol made by Applied Biosystems. An automated protein sequence analysis was carried out on an Applied Biosystems Model 470A gas-liquid phase protein sequencer connected on-line to an Applied Biosystems Model 120A HPLC [1, 3].

RESULTS AND DISCUSSION

The electrophoretic pattern of serum proteins from *R. nigromaculata* and *R. brevipoda* revealed four distinct bands, as shown in Figure. 1. These four bands were previously designated A, B, C and D on the basis of their mobility on SGE [5]. The biggest band A is albumin [5]. There were two bands at the position of A or C in the electrophoretic pattern of serum proteins from a hybrid between these two species (Fig. 1; lane 2). However, only one band of A or C appeared in the serum of *R. nigromaculata* and *R. brevipoda* (Fig. 1; lanes 1 and 3). Therefore, two proteins corresponding to the band A or C must be a product from a codominant allele on the respective chromosome.

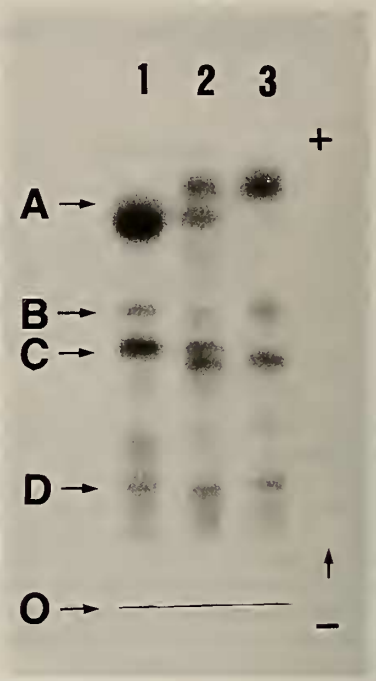

FIG. 1. Electrophoretic pattern of serum proteins of mature frogs on SGE. Proteins in the serum of two species of mature Japanese pond frogs were electrophoresed on a 12% starch-gel and stained with amido black as described in MATERIALS AND METHODS. Letters on the left side of the panel indicate protein A (A), protein B (B), protein C (C), protein D (D) and the original position before SGE (O), respectively. Proteins were mobilized from a cathode (−) to an anode (+) as indicated by an arrow. Lane 1, a male *R. brevipoda*; lane 2, a female hybrid between two species and lane 3, a male *R. nigromaculata*.

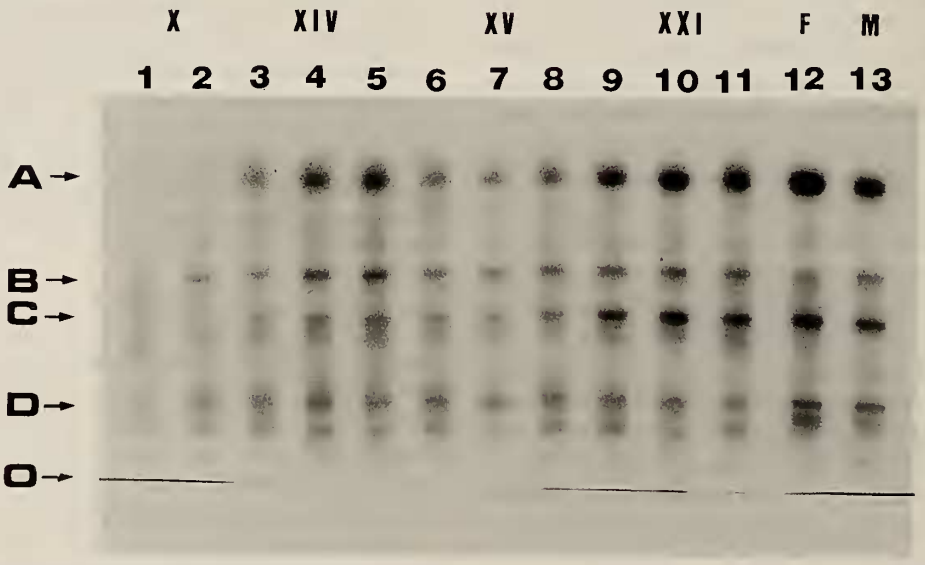

FIG. 2. Developmental pattern of serum proteins of *R. nigromaculata* on SGE. Proteins in the serum from various stages of *R. nigromaculata* were electrophoresed and stained with amido black. See the legend to Figure 1 for details. Letters at the top designate developmental stages. Lanes 1–2, stage X (X); lanes 3–5, stage XIV (XIV); lanes 6–8, stage XV (XV); lanes 9–11, stage XXI (XXI); lane 12, a mature female (F), and lane 13, a mature male (M).

Next, the developmental pattern of serum proteins of *R. nigromaculata* was determined. Five bands were observed at all the stages examined except for stage X (Fig. 2), while four bands were observed in Fig. 2. The appearance of 4 or 5 bands in the electrophoretic pattern probably depends on whether band D is a singlet or doublet (see Figs. 1 and 2). Nishioka *et al.* [5, 6] also found that the appearance of a 5 band pattern, the 5th band having the slowest mobility, depended on the particular blood sample. Hence, the amount of protein in this 5th band probably varies from individual to individual. Protein C did not appear clearly at stage X, while band B did (Fig. 2). Band A (albumin) was also very faint at this stage. Furthermore, protein C was not the second richest protein in the serum of *R. niguromaculata* before stage XIV, but at stage XXI. The pattern at stage XXI was the same as that in the adult (Fig. 2).

The developmental change in body weights of *R. nigromaculata* was depicted in Figure 3. The body weight of this species rapidly increases after stage X and declines beyond stage XIX. The appearance of protein C and albumin in the serum is probably associated with rapid growth in tadpoles of *R. nigromaculata*.

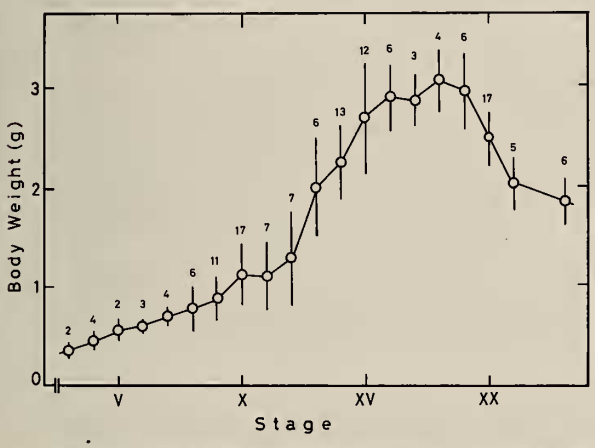

Fig. 3. Developmental change in body weights of *R. nigromaculata*. Body weights of tadpoles of *R. nigromaculata* during early development were determined. Bars indicate the mean ± SD. Values on the top of bars represent the number of animals used.

When protein C from the serum of *R. brevipoda* was run on SGE, extracted with the SDS sample buffer and then run on SDS-PAGE, only one band with Mr = 180 kD was observed (Fig. 4; lane b). The Mr of protein C from *R. nigromaculata* was also 180 kD when estimated on SDS-PAGE (data not shown). The abundance of this protein appeared second among serum proteins of mature frogs (Fig. 4; lane a), which was compatible with the result obtained from SGE (see Fig. 1). The most abundant protein with Mr = 74 kD seemed to be albumin, because it immunoreacted with sheep antisera raised against *Xenopus laevis* albumin on an immunoblot analysis (data not shown).

To identify protein C, the NH_2-terminal amino acid sequence was determined. As shown in Fig. 5, the sequence

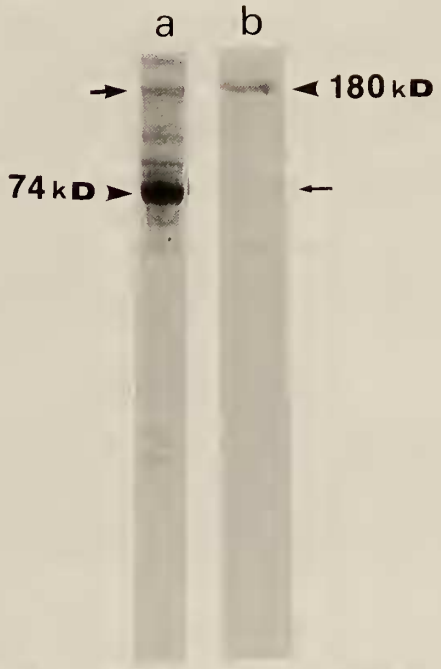

Fig. 4. Profile of proteins on SDS-PAGE. Crude serum (a) and protein C (b) of *R. brevipoda* obtained after SGE were then run on SDS-PAGE and stained with Coomassie brilliant blue R (Sigma) [2]. A large arrow indicates protein C in the crude serum. The richest protein with Mr = 74 kD is likely to be albumin (see Results). A small arrow indicates a position of albumin that migrates on SDS-PAGE. Thirty and three μg of proteins were loaded for lanes a and b, respectively.

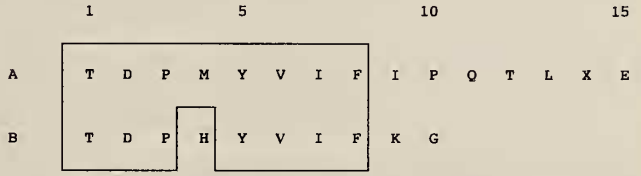

Fig. 5. The NH_2-terminal amino acid sequence of protein C in sera of two species, *R. nigromaculata* (A) and *R. brevipoda* (B). The homologous area is blocked.

of the first 15 amino acids of protein C from *R. nigromaculata* and the first 10 amino acids from *R. brevipoda* revealed that they were not identical, but probably in the same family. The heterogeneity of amino acid compositions between two proteins could be one reason why they showed different mobility on SGE (see Fig. 1), as they have the same Mr of 180 kD. Comparison of these sequences with the existing protein data base held by GenBank showed no significant identity with any previously described protein. Possible reasons for this include; (1) the molecular size of protein C is fairly large, (2) efforts to determine the amino acid sequence of such proteins deduced from their cDNAs have been made mainly in studies of animals other than amphibians, and (3) there should naturally be a substantial difference in amino acid composition of proteins from frogs and other animals. Taking all these possible factors into consideration, it is

conceivable that no significant homology of protein C to others could be observed, even when the field was narrowed to serum proteins.

It is of great interest to note that protein C appears in the serum of the frog, *R. nigromaculata* when body weight increases rapidly. Protein C must have an imporant function(s) in the serum of this species, but there is no evidence for this at the present time. To allow understanding of its molecular structure and physiological role(s), the nucleotide sequence of its cDNA should be determined right away.

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